AD_____

GRANT NUMBER DAMD17-97-1-7273

TITLE: The Role of the Complement Inhibitor CD59 on Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Stephen Tomlinson, Ph.D.

CONTRACTING ORGANIZATION: New York University
New York, New York 10016

REPORT DATE: October 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1998	Annual (15 Sep 97 - 14 Sep 98)

4. TITLE AND SUBTITLE

The Role of the Complement Inhibitor CD59 on Breast Cancer Cells

5. FUNDING NUMBERS

DAMD17-97-1-7273

6. AUTHOR(S)

Tomlinson, Stephen, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

New York University Medical Center
New York, New York 10016

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

19990209 117

13. ABSTRACT *(Maximum 200 words)*

It is proposed that reversing the effects of CD59, a tumor cell expressed complement inhibitor, will allow effective immune-mediated clearance of tumor cells and improve prospects for successful immunotherapy. This study investigates the role of complement and CD59 in tumorigenesis. CD59 function is species selective, and an appropriate rat model utilizing human tumor cells expressing rat CD59 will be developed.

We have: 1. Confirmed that CD59 is important in protecting human tumor cells from complement-mediated lysis. 2.Identified several anti-breast tumor-specific antibodies that target active complement to breast cancer cell lines. 3. Determined growth conditions for breast cancer cell lines in nude rats, and 4. Further defined the species selective active site of human CD59 for future design of CD59-inhibitory peptides.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Complement, CD59, antibody,, immunotherapy, peptide	36
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

ST ✓ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

ST ✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

ST ✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

ST ✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

S Tan 8/31/98

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

Hypothesis

Complement effector systems involved in the immune response to tumor cells include amplification of inflammatory response, recruitment of immune effector cells and direct and NK cell mediated cytolysis. It is hypothesized that complement regulatory proteins expressed on the tumor cell surface promote tumorigenesis and present a barrier to effective complement-mediated immunotherapy. We propose that reversing the effects of tumor-expressed complement inhibitors will allow effective immune-mediated clearance of tumor cells and improve prospects for successful immunotherapy. *The current project is focussed on the study of the complement inhibitory protein, CD59.*

Background

Complement is one of the major effector mechanisms of the immune system and its activation results in the formation of the amplification C3/C5 convertases, which cleave C5 to initiate the formation of the membrane attack complex (MAC or C5b-9), usually on the activating cell surface. The cytolytic MAC is formed from the sequential assembly of the soluble plasma proteins, C5b, C6, C7, C8 and C9.

The activation of complement on a tumor or normal host cell can occur following nonspecific, antibody-independent complement deposition via the alternative pathway (1). Specific complement deposition can also occur on a tumor cell following the binding of antibodies to tumor cell surface proteins (such tumor-specific antibodies are often found in patient's serum). In either case, complement activation on tumor (and normal) cells is controlled by various membrane proteins; decay-accelerating factor (DAF), membrane cofactor protein (MCP) and complement receptor 1 (CR1). These proteins inhibit C3/C5 convertase formation. Control of cytolytic MAC formation on host cell membranes is provided by CD59, a cell surface glycoprotein that binds to C8 and C9 in the assembling MAC and prevents membrane insertion.

An important feature of complement regulatory proteins (particularly CD59) is their species selectivity. For eg., human CD59 inhibits MAC formation by human and primate complement, but not by rodent complement (2-4). Membrane inhibitors of complement protect tumor cells from cytolytic complement attack *in vitro*. CD59 and usually DAF and/or MCP are expressed by virtually all breast and other primary tumors and tumor cell lines that have been examined (5-8). CD59 neutralization *in vitro* by anti-CD59 mAbs enhance complement-mediated lysis of breast tumor cells (5). Thus, effective lysis of breast tumor cells by complement *in vitro* requires that their resistance to complement be overcome, an important consideration for complement-dependent immunotherapy using mAbs. It is now clear that cancer specific antibodies are produced by patients, and breast tumor-specific or overexpressed antigens (eg. HER2/neu, MUC1) have been identified (9-13).

BODY

Some of the data described below has been submitted to Clinical and Experimental Immunology for publication (accepted subject to minor revision) and the manuscript is included.

TASK 1: Months 0-6: IN VITRO EXPERIMENTS: Confirmation of the role of CD59 in conferring protection against antibody-targeted complement lysis of tumor cells. Will transfect human tumor cell lines with rat CD59 and select expressing populations. Will determine if transfected cells have increased resistance to rat complement.

This task has been completed (and extended to include mouse CD59)

Results

Complement-mediated lysis of MCF7 cells expressing rodent CD59

MCF7 were transfected with rat or mouse CD59 cDNA, and cell populations stably expressing high levels of recombinant rodent CD59 were isolated by cell sorting (fig. 1). Transfected cell populations were then

tested for their susceptibility to complement-mediated lysis to determine whether expression of rodent CD59 correlated with increased resistance to rodent complement. Untransfected MCF7 cells are relatively resistant to lysis by homologous human complement, but are effectively lysed by both rat and mouse complement (fig. 2, and see also fig. 4 below). The expression of either rat or mouse CD59 on MCF7 cells however, protected them from lysis by rat and mouse complement, respectively (fig. 2). MCF7 cells expressing rat CD59 were almost totally resistant to lysis by 40% rat complement. The increased rat complement resistance of rat CD59 transfected MCF7 cells was reversed by the addition of anti-rat CD59 blocking mAb 6D1 (not shown), thus confirming that the heterologously expressed rodent CD59 is responsible for providing the observed protection from rodent complement-mediated lysis. It is possible that an anti-CD59 antibody could increase cell lysis by fixing complement, but it has been demonstrated previously that mAb 6D1 alone does not cause increased complement-mediated cell lysis by activating complement (14). These data demonstrate the relative activities of each CD59 protein against heterologous sera, and data is relevant to establishing rodent models for the study of complement and complement inhibitors in tumor growth and control.

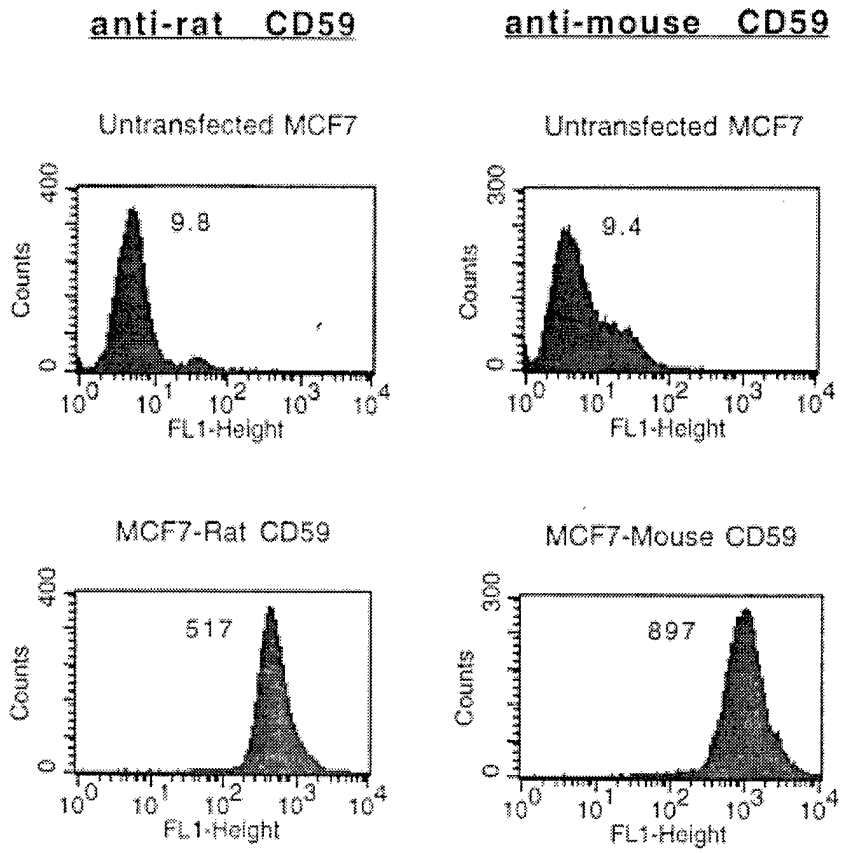


Fig. 1. Expression of rodent CD59 by transfected MCF7. Stably transfected homogenous populations of MCF7 cells expressing either rat or mouse CD59 were isolated by several rounds of cell sorting. Figure shows flow cytometric analysis of sorted populations. Cells were stained by immunofluorescence using anti-rat CD59 monoclonal antibody (6D1) or rabbit anti-mouse CD59 polyclonal antibody. Note that Immunofluorescence is not quantitative relative to the different CD59 proteins. Histograms of the relative mean fluorescence intensities are shown.

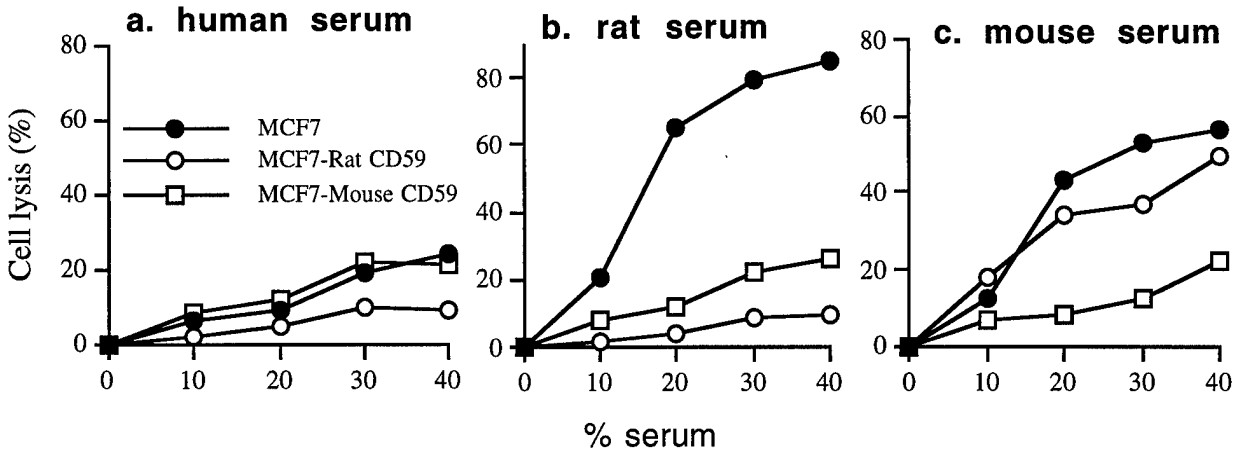


Fig. 2. Complement-mediated lysis of MCF7 cells expressing rat and mouse CD59. Control and transfected MCF7 cells were sensitized to complement by preincubation in 15% anti-MCF7 antiserum. Sensitized cells were exposed to different concentrations of either human (a), rat (b) or mouse (c) serum, and lysis determined. The omission of either sensitizing antibody or of serum in cell lysis assays resulted in a background lysis of less than 10% of test value. Figure shows representative data from 3 different experiments.

Methods

Transfection of MCF7 cells and flow cytometry

cDNA constructs were transfected into 50-75% confluent MCF7 cells using Lipofectamine according to the manufacturers instructions (Gibco). Stable populations of MCF7 cells were isolated by three rounds of cell sorting using anti-rat CD59 or anti-mouse CD59 antibodies as described (15). Analysis of cell surface protein expression was performed by flow cytometry using appropriate antibodies (15).

Cell lysis assays

Complement-mediated cell lysis was determined by both ^{51}Cr release (16) and by microscopic examination following trypan blue staining (17) as described. Both methods gave similar results. Rabbit antisera to MCF7 cell membranes that was used to sensitize MCF7 cells to complement was prepared by standard techniques (18).

Further experimental details are described in a submitted manuscript that is included with this report (19).

Discussion

The demonstration that heterologous (nonhuman) cells transfected with human CD59 display increased resistance to lysis by human complement provided direct and unequivocal evidence that human CD59 inhibits human complement-mediated cell lysis (20,21). The phenomenon of species selective activity allowed us to use a reciprocal approach to determine directly the functional significance of CD59 expressed on human breast tumor cells. Data generated is relevant to establishing rodent models for the study of complement and complement inhibitors in tumor growth and control.

TASK 2: Months 0-12: IN VITRO EXPERIMENTS: Determination of the effect or rat complement on human breast cancer cells. First, different breast tumor cell lines will be screened for CD59 expression. Sensitivity of CD59 positive cells to rat serum will be assayed. Cells will be sensitized to complement using tumor cell specific antibodies. Will repeat experiments using purified complement components to show if CD59 is inhibiting rat complement protein C9.

This task has been performed using the MCF7 cell line, and studies using other cell lines are near completion (preliminary data not shown indicate that the breast cancer cell lines BT474, T47D and SKBr3 show similar cell lysis characteristics as MCF7).

Results

Expression of endogenous membrane complement inhibitors on MCF7

Flow cytometric analysis confirmed the endogenous expression of the human membrane-bound complement inhibitors CD59, DAF and MCP on MCF7 cells (fig. 3). We have also determined that DAF, MCP and CD59 is similarly expressed on the breast cancer cell lines BT474, T47D and SKBr3 (data not shown).

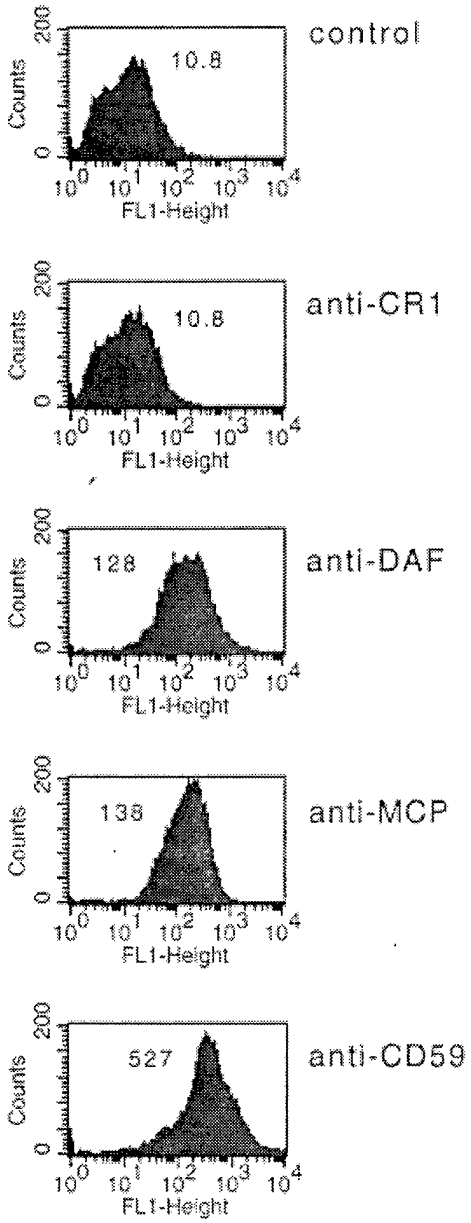


Fig. 3. Endogenous expression of complement inhibitory proteins by MCF7. Cells were stained by immunofluorescence using monoclonal antibodies to human CD59 (YTH53.1), MCP (M75), DAF (1A10), and CR1 (57F) as primary antibodies. Isotype matched antibodies of irrelevant specificity were used for controls. Relative fluorescence resulting from all control antibodies was below 12. Staining with a representative control antibody is shown. Histograms of the relative mean fluorescence intensities are shown.

Lysis of MCF7 cells by human and rodent sera
Rabbit antiserum raised against MCF7 cell membranes effectively sensitized MCF7 cells to lysis by rat and mouse complement. However, antibody sensitized MCF7 cells were significantly more resistant to lysis by human complement (fig. 4). At a concentration of rat serum giving half-maximal lysis, the equivalent human serum concentration resulted in 5-fold less lysis. The relative sensitivity of MCF7 to lysis by rodent, but not human complement, is indicative of species selective complement inhibition by endogenous membrane-bound inhibitors.

Previous data have shown that human CD59 does not function effectively against rat complement (4), and the data shown in fig. 4 indicate that endogenous expression of DAF and MCP on MCF7 also does not effectively protect the cells from lysis by rat or mouse complement (see also fig. 2 above).

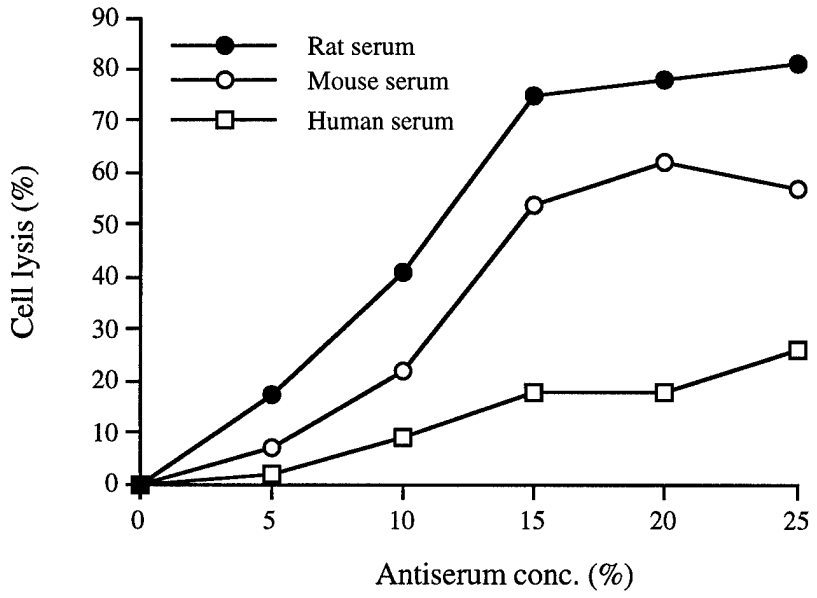


Fig. 4. Complement-mediated lysis of MCF7 cells. MCF7 cells were sensitized to complement by preincubation in the indicated concentrations of anti-MCF7 membrane rabbit antiserum. Sensitized cells were washed in media, exposed to 25% of either human, rat or mouse complement (37 ^{o}C/60 min), and cell lysis determined. The omission of either sensitizing antibody or of serum in cell lysis assays resulted in a background lysis of less than 10% of test value. Figure shows representative data from 3 separate experiments.

Methods
Applicable methods are described above (task 1) and in figure legends.

TASK 3: Months 6-18: IN VITRO EXPERIMENTS: Targeting CD59 inhibitory antibodies to breast tumor cells. Will confirm that rat adenocarcinoma 13762 cells express CD59. Then confirm their susceptibility to rat complement after neutralization of rat CD59. If successful, will isolate 13762-specific antibodies and attempt to target anti-rat CD59 mAbs to 13762 cell surface by means of 13762-specific antibodies and biotin-avidin bridges.

We have determined that rat adenocarcinoma 13762 cells express high levels of CD59 (and also Crry, a complement inhibitor of activation) (fig. 5). Functional studies to determine the susceptibility of 13762 cells to rat complement after neutralization of rat CD59 have just been initiated.

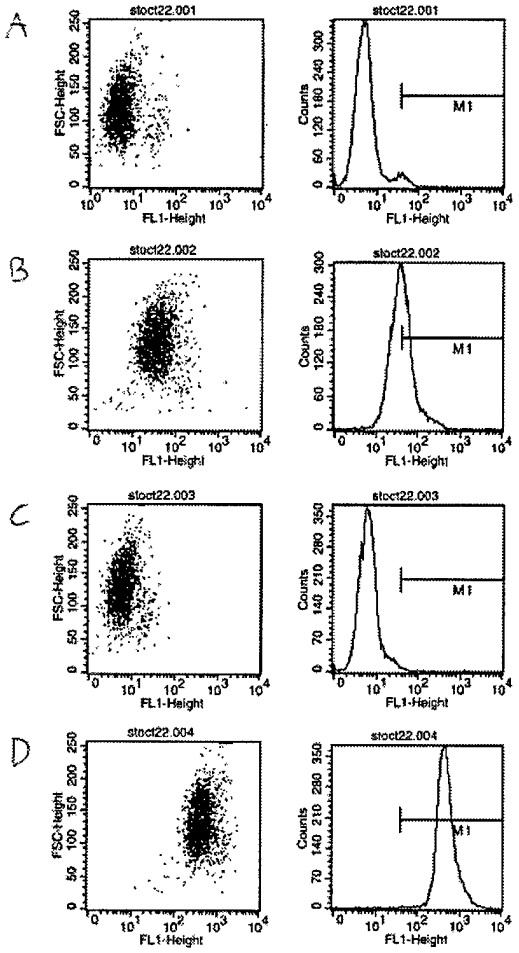


Figure 5. Endogenous expression of CD59 and Crry by rat adenocarcinoma 13762 cells. Cells were stained by immunofluorescence using monoclonal antibodies to rat CD59 (B) or rat Crry (D) as primary antibodies. Isotype matched antibodies of irrelevant specificity were used for controls (A and C).

Methods

Rat CD59 and rat Crry expression on 13762 cells was determined by flow cytometry by standard procedures (4).

Discussion

Data obtained will now allow progression onto the more relevant functional experiments that constitute part of this task.

TASK 4: Months 6-24: IN VITRO: Will test rat and human breast tumor cell lines transfected with HER2 and rat CD59, respectively, for sensitivity to rat complement. Will assess ability of tumor specific antigens to target transfected cells. Will attempt to target anti-rat CD59 antibodies to transfected cell surface by means of tumor specific antibody and biotin-avidin bridge.

Progress on this task has been made. This task is related to tasks 1 and 2, and data presented above is relevant. Two breast cancer cell lines have been transfected with rat CD59: MCF7 (see fig. 2 for functional data) and BT474 (not shown). Their susceptibility to rat complement has been determined. Cell lysis of

MCF7 and rat CD59 transfected MCF7 is shown in figs 2 and 4. The lysis of untransfected and transfected BT474 by rat complement (using an anti-BT474 membrane complement-sensitizing antiserum) was the same as that for MCF7 and is not shown.

BT474 is a HER2 positive cell line. MCF7 is a MUC1 positive cell line. We have shown that certain antibodies against the breast tumor-specific antigens HER2 and MUC1 are able to target breast cancer cell lines and activate complement (fig. 6).

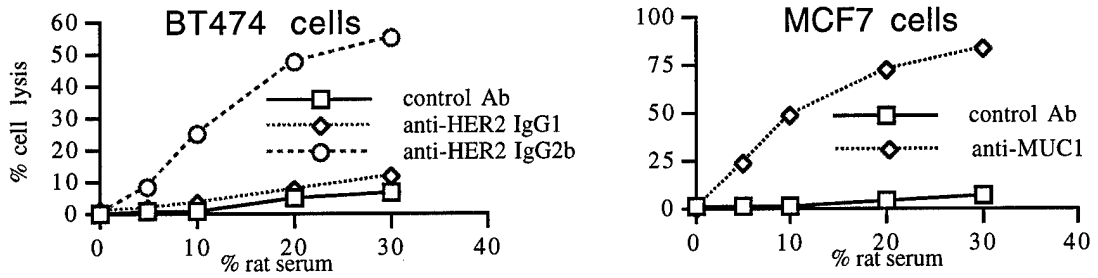


Fig 6. Breast tumor cell lines can be sensitized to heterologous complement by antibody recognizing tumor-specific (overexpressed) antigen. Standard assay procedures were followed (see above). The anti-MUC1 antibody source used was rabbit polyclonal antiserum. The anti-HER2 antibodies were purified mAbs used at 20 ug/ml.

An IgM monoclonal antibody directed against the breast cancer-associated antigen MUC1 (BC3,(22)) also sensitized MCF7 cells to lysis by rat complement, but was less effective than the polyclonal antiserum shown above (data not shown).

Discussion

The presented data indicate that endogenous CD59 expressed on human tumor cells implanted into rodents is unlikely to provide effective protection against complement attack when tumors are targeted by complement activating antibodies. The relative ineffectiveness of human CD59 against rat and mouse complement presents a serious hindrance for studies aimed at determining the protective role of CD59 (and other complement inhibitors) in rodent hosts bearing human cancers. The current data establishes the feasibility of using human cancer cells expressing rodent CD59 to show, in vivo, the regulatory effects of CD59 on complement-mediated tumor cell lysis.

TASK 5: Months 0-36: Will use molecular modelling techniques to determine C9 peptide ligand for CD59 binding, and determine three dimensional structure of the CD59-C9 peptide ligand complex.

We have better defined the binding site for C9 on the CD59 protein, and these studies will greatly assist in aim of the determination of the three dimensional structure of the CD59-C9 peptide ligand complex.

Results

Our previous functional analysis of chimeric human/rat CD59 proteins indicated that the residues responsible for the species selective function of human CD59 map to a region contained between positions 40-66 in the primary structure (4). By comparative analysis of rat and human CD59 models and by mutational analysis of candidate residues, we have now identified individual residues within the 40-66 region that confer species selective function on human CD59. Individual human to rat substitutions F47A, T51L, R55E and K65Q each produced a mutant human CD59 protein with enhanced rat complement inhibitory activity. Substitutions of all other nonconserved residues between positions 40-66 did not effect species selective function. The F47A, T51L, R55E substitutions were the most effective, and interestingly the side chains of these residues are all located on the same face of the short helix of CD59 (fig. 7).

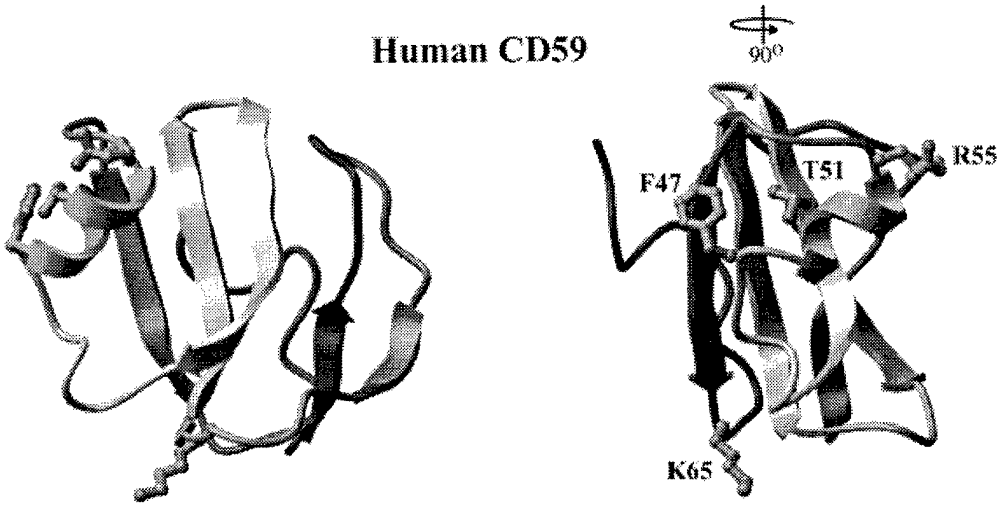


Fig. 7. Ribbon diagram of human CD59 showing residues involved in species selective function.

Methods.
Human CD59 residues were substituted for rat residues by PCR mutagenesis procedures. The mutatnt proteins were expressed on the surface of CHO cells, and the susceptibility of the transfected CHO cells to human and rat complement determined. Human CD59 does not inhibit rat complement, and the acquisition of rat complement inhibitory activity by the substitution of a rat residue indicates a role for that residue in species selective function. The experimental procedures are described in detail in ref (4).

TASK 6: Months 6-36: IN VIVO: Continuation of task 1. Determine which human breast cancer cell lines grow in nude rats (about 20 rats required). Will Use cell line that developed tumors and that has been successfully transfected with rat CD59 to seed nude rats. Will then determine the effect of tumor-specific antibodies on growth of these cells in rats (about 40 rats required).

We have established conditions for estrogen-supplemented growth of MCF7 and BT474 cell lines in Rowett nude rats. Between 5 x 10^5 and 1 x 10^7 cells injected per site with matrigel produce tumors. A full dose response study of untransfected cells is underway. The cell line SKBR3 did not grow in nude rats under the conditions tested (up to 1 x 10^7 cells injected per site with and without matrigel). Other cell lines are under investigation.

TASK 7: Months 12-30: IN VIVO: Continuation of task 2. Will determine if tumor-specific antibodies can elimate or reduce human breast cancer cell growth in rats (about 30 rats). To confirm role of CD59 and complement in any reduction in tumor growth that is observed, rats will be depleted of complement and re-tested (about 20 rats).

Studies not yet initiated.

TASK 8: Months 18-36: IN VIVO: Continuation of tasks 3 and 4. Tumors will be grown in rats using cells described above. Will determine whether anti-CD59 antibodies can be targeted to tumors using tumor-specific antibodies and biotin-avidin bridges (about 50 rats).
Studies not yet initiated

CONCLUSIONS

In conclusion, our data strengthen the hypothesis that the modulation of CD59 activity on a tumor cell surface will provide an effective therapy when combined with complement-activating anti-tumor antibodies. Neutralization of CD59 (or other complement regulatory proteins) may also enhance a normally ineffective cytolytic humoral immune response. These hypotheses now need to be tested in vivo. To this end, the current data define important parameters necessary for establishing rodent models designed to evaluate the role of complement and CD59 in the growth and control of human cancer.

REFERENCES

1. Law, S. K. A. and K. B. M. Reid. 1988. Complement. IRL Press, Oxford.

2. Rollins, S. A., J. I. Zhao, H. Ninomiya, and P. J. Sims. 1991. Inhibition of homologous complement by CD59 is mediated by a species-selective recognition conferred through binding to C8 within C5b-8 or C9 within C5b-9. *J. Immunol.* 146:2345-2351.

3. Lockert, D. H., K. M. Kaufman, C-P. Chang, T. Huesler, J. M. Sodetz, and P. J. Sims. 1995. Identity of the segment of human complement C8 recognized by complement regulatory protein CD59. *J. Biol. Chem.* 270:19723-19728.

4. Yu, J., S. Dong, N. K. Rushmere, B. P. Morgan, R. Abagyan, and S. Tomlinson. 1997. Mapping the regions of the complement inhibitor CD59 responsible for its species selectivity. *Biochem.* 36:9423-9428.

5. Hakulinen, J. and S. Meri. 1994. Expression and function of the complement membrane attack complex inhibitor protectin (CD59) on human breast cancer cells. *Lab. Invest.* 71:820-827.

6. Brasoveanu, L. I., M. Altomonte, A. Gloghini, E. Fonsatti, S. Coral, A. Gasparollo, R. Montagner, I. Cattarossi, C. Simonelli, A. Cattelan, V. Attadia, A. Carbone, and M. Maio. 1995. Expression of protectin (CD59) in human melanoma and its functional role in cell- and complement-mediated cytotoxicity. *Int. J. Cancer* 61:548-556.

7. Bjorge, L., C. A. Vedeler, E. Ulvestad, and R. Matre. 1994. Expression and function of CD59 on colonic adenocarcinoma cells. *Eur. J. Immunol.* 24:1597-1603.

8. Yamakawa, M., K. Yamada, T. Tsuge, H. Ohrui, T. Ogata, M. Dobashi, and Y. Imai. 1994. Protection of thyroid cancer cells by complement-regulatory factors. *cancer* 73:2808-2817.

9. Hollingsworth, M. A. and R. S. Metzgar. 1985. Monoclonal antibodies in cancer. Humana Press, Clifton, NJ.

10. Bianchi, E., R. Cohen, A. Thor, I. Todd, I. Mizukami, B. Lawrence, M. Ljung, M. Sherman, and H. Smith. 1994. The urokinase receptor is expressed in invasive breast cancer but not in normal breast tissue. *Cancer Res.* 54:861-866.

11. Devine, P. and I. McKenzie. 1992. Mucins:structure, function and associations with malignancy. *BioEssays* 14:619-625.

12. Dissis, M., E. Calenoff, G. McLaughlin, A. Murphy, W. Chen, B. Groner, M. Jeschke, N. Lydon, E. McGlynn, R. Livingston, R. Moe, and M. Cheever. 1994. Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. *Cancer Res.* 54:16-20.

13. Kotera, Y., J. Fontenot, G. Pecher, R. Metzger, and O. Finn. 1994. Humoral immunity against a tandem repeat epitope of human mucin MUC-1 in sera from breast, pancreatic, and colon cancer patients. *Cancer Res.* 54:2856-2860.

14. Hughes, T. R., S. J. Piddlesden, J. D. Williams, R. A. Harrison, and B. P. Morgan. 1992. Isolation and characterization of a membrane protein from rat erythrocytes which inhibits lysis by the membrane attack complex of rat complement. *Biochem. J.* 284:169-176.

15. Yu, J., R. A. Abagyan, S. Dong, A. Gilbert, V. Nussenzweig, and S. Tomlinson. 1997. Mapping the active site of CD59. *J. Exp. Med.* 185:745-753.

16. Helfand, S. C., J. A. Hank, J. Gan, and P. M. Sondel. 1996. Lysis of human tumor cell lines by canine complement plus monoclonal antiganglioside antibodies or natural cannine xenoantibodies. *Cell. Immunol.* 167:99-107.

17. Rushmere, N. K., S. Tomlinson, and B. P. Morgan. 1997. Expression of rat CD59: functional analysis confirms lack of species specificity and reveals that glycosylation is not required for function. *Immunol.* 90:640-646.

18. Harlow, E. and D. Lane. 1988. Antibodies. A laboratory manual. Cold Spring Harbor Laboratory, New York.

19. Yu, J., T. Caragine, S. Chen, B. P. Morgan, A. F. Frey, and S. Tomlinson. 1998. Protection of human breast cancer cells from complement-mediated lysis by expression of heterologous CD59. *Clin. Exp. Immunol.* Submitted:

20. Walsh, L. A., M. Tone, and H. Waldmann. 1991. Transfection of human CD59 complementary cDNA into rat cells confers resistance to human complement. *Eur. J. Immunol.* 21:847-850.

21. Zhao, J., S. A. Rollins, S. E. Maher, A. L. M. Bothwell, and P. J. Sims. 1991. Amplified gene expression in CD59-transfected chinese hamster ovary cells confers protection agaisnt the membrane attack complex of human complement. *J. Biol. Chem.* 266:13418-13422.

22. Xing, P. X., J. J. Tjandra, S. A. Stacker, J. G. Teh, P. J. McLaughlin, and I. F. C. McKenzie. 1989. Monoclonal antibodies reactive with mucin expressed in breast cancer. *Immunol. Cell. Biol.* 67:183-195.

APPENDICES

Yu, J., Caragine, T,. Chen, S., Morgan, B. P., Frey, A. and Tomlinson, S. (1998) Protection of human breast cancer cells from complement-mediated lysis by expression of heterologous CD59. *Clin. Exp. Immunol. (Submitted).*

Protection of human breast cancer cells from complement-mediated lysis by expression of heterologous CD59.

Short title: Complement-mediated lysis of tumor cells

J. Yu, T. Caragine, S. Chen, B. P. Morgan, A. B. Frey & S. Tomlinson*

J. Yu, T. Caragine, S. Chen, S. Tomlinson, New York University Medical Center, Department of Pathology, 550 First Avenue, New York, NY 10016
A. Frey, New York University Medical Center, Department of Cell biology, 550 First Avenue, New York, NY 10016
B. P. Morgan, Department of Medical Biochemistry, University of Wales College of Medicine, Heath Park, Cardiff, UK.

*Correspondance

Stephen Tomlinson
New York University Medical Center
Department of Pathology, MSB 127
550 First Avenue
New York, NY 10016

Telephone (212) 263 8514
FAX (212) 263 8179
email: tomlis01@popmail.med.nyu.edu

Keywords: CD59, complement, breast cancer, anti-tumor antibody.

SUMMARY

CD59, decay accelerating factor (DAF) and membrane cofactor protein (MCP) are widely expressed cell surface glycoproteins that protect host cells from the effects of homologous complement attack. Complement inhibitory activity of these proteins is species selective. We show that the human breast cancer cell line MCF7 is relatively resistant to lysis by human complement, but is effectively lysed by rat or mouse complement. CD59, DAF and MCP were all shown to be expressed by MCF7. The species selective nature of CD59 activity was used to demonstrate directly the effectiveness of CD59 at protecting cancer cells from complement-mediated lysis. cDNA's encoding rat and mouse CD59 were separately transfected into MCF7 cells, and cell populations expressing high levels of the rodent CD59 were isolated by cell sorting. Data show that rat and mouse CD59 were highly effective at protecting transfected MCF7 cells from lysis by rat and mouse complement, respectively. Data further reveals that rat CD59 is not effective against mouse complement, whereas mouse CD59 is effective against both mouse and rat complement. These studies establish a model system for relevant in vivo studies aimed at determining the effect of complement regulation on tumorigenesis, and show that for effective inmunotherapy using complement-activating anti-tumor antibodies, the neutralization of CD59 and/or other complement inhibitory molecules will likely be required.

Key words CD59, complement, complement inhibitor, antibody-mediated lysis.

INTRODUCTION

Complement is one of the major effector mechanisms of the immune system and its activation results in the formation of the C3/C5 convertases, which cleave C5 to initiate the formation of the membrane attack complex (MAC or C5b-9). The cytolytic MAC is formed from the sequential assembly of the soluble plasma proteins, C5, C6, C7, C8 and C9. Complement *activation* on host cells is controlled by various membrane proteins which inhibit C3/C5 convertase formation; decay-accelerating factor (DAF), membrane cofactor protein (MCP) and complement receptor 1 (CR1). Control of cytolytic MAC formation (the terminal complement pathway) on host cell membranes is provided by CD59, a widely distributed cell surface glycoprotein that binds to C8 and C9 in the assembling MAC. For review of complement inhibitory membrane proteins, see [1].

CD59 and usually DAF and/or MCP are expressed by virtually all breast and other primary tumors and tumor cell lines that have been examined, and several studies have reported the upregulation of complement inhibitory proteins on tumor cells [2-8]. Neutralization of complement regulatory proteins on the surface of tumor cells by antibodies significantly increases their susceptibility to complement-mediated lysis in vitro [2,3,5,9,10]. The only relevant in vivo experiment reported to date, shows that pretreatment of rat tumor cells with an antibody that blocks the function of a rat complement inhibitor (Crry/p65), substantially increases survival time of recipient rats after transplantation of treated tumors [11]. There is thus very good evidence to support the hypothesis that tumor-expressed complement inhibitory proteins play an important role in promoting tumor growth by inhibiting complement activation and cytolysis. A significant contributing factor in the lack of success of complement-activating mAbs in clinical trials to date may therefore be the presence of complement inhibitors on the tumor cell surface. Also, inhibition of tumor-expressed complement regulators may enhance an ineffective cytolytic humoral immune response against tumor cells in therapy which does not involve administration of exogenous activator antibodies.

An important feature of membrane complement regulatory proteins is their species selective inhibitory activity [12-18]. These proteins display significant variations in their effectiveness at inhibiting heterologous complement. Thus, the role of complement inhibitors expressed on human cancer cells is difficult to assess in rodent models, since human inhibitors may have limited function against rodent complement. Here we demonstrate directly for the first time the protective role that CD59 provides to a human breast cancer cell. We have determined patterns of species selective activity of endogenous human complement inhibitors, and of rat and mouse CD59 expressed in a human tumor cell line MCF7. These data will permit the planning of meaningful in vivo studies aimed at determining the role of CD59 in promoting tumor growth.

MATERIALS AND METHODS

Cells and DNA

The human breast cancer cell line MCF7 was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in Eagle's modified essential medium (EMEM) supplemented with 10% FCS, 0.1% non-essential amino acids and bovine insulin (10 ug/ml). cDNA encoding rat [19] and mouse [20] CD59 was subcloned into the mammalian expression vectors pCDNA3 (Invitrogen, Carlsbad, CA) and pDR2Ef1a [21], respectively. pDR2Ef1a was a gift from Dr. I. Anegon (Nantes, France). Stably transfected MCF7 cell populations were selected following the cultivation of cells in the presence of G418 (pCDNA3) or hygromycin (pDR2Ef1a).

Antibodies and complement

Rabbit antisera to MCF7 cell membranes that was used to sensitize MCF7 cells to complement was prepared by standard techniques [22]. Flow cytometric analysis of MCF7 cells using anti-MCF7 antiserum gave a positive signal at a dilution of 1:200. Cell membranes were prepared by Dounce

homogenization of cells in hypotonic media (10 mM sodium phosphate, pH 8) and subcellular fractionation to remove nuclei and mitochondria. Anti-rat CD59 mAb 6D1 [23], anti-mouse CD59 polyclonal antibody [20] and anti-DAF mAb 1A10 [24] were described previously. Anti-MCP mAB M75 [25] and anti-human CD59 mAb YTH53.1 [26] were gifts from Drs. D. Lublin (St. Louis, MO) and H. Waldmann (Oxford, UK), respectively. FITC-conjugated antibodies used for flow cytometry were purchased from Sigma (St. Louis, MO). Normal human serum (NHS) was obtained from the blood of healthy volunteers in the laboratory. Mouse serum was prepared from the blood of BUB/BnJ mice (Jackson Laboratories, Bar Harbor, ME). Mouse blood was collected by heart puncture, and sera processed after clotting for three hours on ice. Freshly collected rat serum was purchased from Cocalico Biologicals (Reamstown, PA). All sera were stored in aliquots at -70 ^{o}C until use.

Transfection of MCF7 cells and flow cytometry

cDNA constructs were transfected into 50-75% confluent MCF7 cells using Lipofectamine according to the manufacturers instructions (Gibco). Stable populations of MCF7 cells were isolated by three rounds of cell sorting using anti-rat CD59 or anti-mouse CD59 antibodies as described [27]. Analysis of cell surface protein expression was performed by flow cytometry using appropriate antibodies [27].

Cell lysis assays

Complement-mediated cell lysis was determined by both ^{51}Cr release [28] and by microscopic examination following trypan blue staining [29] as described. Both methods gave similar results. Briefly, MCF7 cells were detached by a 3 min/25 ^{o}C treatment with trypsin/EDTA (Gibco), washed once and resuspended in EMEM/10% heat inactivated FCS. For the trypan blue exclusion assay, cells were resuspended to 1 x 10^6/ml. For ^{51}Cr release assay, cells were preloaded at a concentration of 1 x 10^7/ml (2h/37 ^{o}C), washed in complete media and resuspended to 1 x 10^6/ml.

Rabbit anti-MCF7 cell membrane antiserum diluted in EMEM/10% FCS was added and the cells incubated on ice for 30 min. Cells were centrifuged and resuspended to 1 x 10^6/ml in EMEM/10% FCS. Equal volumes of cells and serum dilutions were incubated for 60 min. at 37 °C, and cell lysis determined. The effect of anti-rat CD59 mAb 6D1 on rat complement-mediated lysis was performed as previously described [29].

RESULTS

Lysis of MCF7 cells by human and heterologous serum

Rabbit antiserum raised against MCF7 cell membranes effectively sensitized MCF7 cells to lysis by rat and mouse complement. However, antibody sensitized MCF7 cells were significantly more resistant to lysis by human complement (fig. 1). At a concentration of rat serum giving half-maximal lysis, the equivalent human serum concentration resulted in 5-fold less lysis. An IgM monoclonal antibody directed against the breast cancer-associated antigen MUC1 (BC3,[30]) also sensitized MCF7 cells to lysis by rat complement, but was less effective than the polyclonal antiserum (data not shown).

Expression of endogenous membrane complement inhibitors on MCF7

The relative sensitivity of MCF7 to lysis by rodent, but not human complement, is indicative of species selective complement inhibition by endogenous membrane-bound inhibitors. Flow cytometric analysis confirmed the expression of the membrane-bound complement inhibitors CD59, DAF and MCP on MCF7 cells (fig. 2). Previous data have shown that human CD59 does not function effectively against rat complement [18], and the data shown here indicate that endogenous expression of DAF and MCP on MCF7 does not effectively protect the cells from lysis by rat and mouse complement (figs. 1 and 2).

Complement-mediated lysis of MCF7 cells expressing rodent CD59

The demonstration that heterologous (nonhuman) cells transfected with human CD59 display increased resistance to lysis by human complement provided direct and unequivocal evidence that human CD59 inhibits human complement-mediated cell lysis [31,32]. The phenomenon of species selective activity allowed us to use a reciprocal approach to determine directly the functional significance of CD59 expressed on human breast tumor cells.

MCF7 were transfected with rat or mouse CD59 cDNA, and cell populations stably expressing high levels of recombinant rodent CD59 were isolated by cell sorting (fig. 3). Transfected cell populations were then tested for their susceptibility to complement-mediated lysis to determine whether expression of rodent CD59 correlated with increased resistance to rodent complement. Untransfected MCF7 cells are relatively resistant to lysis by homologous human complement, but are effectively lysed by both rat and mouse complement (figs. 1 and 4). The expression of either rat or mouse CD59 on MCF7 cells however, protected them from lysis by rat and mouse complement, respectively (fig. 4). MCF7 cells expressing rat CD59 were almost totally resistant to lysis by 40% rat complement. The increased rat complement resistance of rat CD59 transfected MCF7 cells was reversed by the addition of anti-rat CD59 blocking mAb 6D1 (not shown), thus confirming that the heterologously expressed rodent CD59 is responsible for providing the observed protection from rodent complement-mediated lysis. It is possible that an anti-CD59 antibody could increase cell lysis by fixing complement, but it has been demonstrated previously that mAb 6D1 alone does not cause increased complement-mediated cell lysis by activating complement [23].

Figure 4 further reveals a pattern of species selective activity for rat and mouse CD59. Rat CD59 effectively protected MCF7 cells from lysis by rat complement (fig. 4b), but not mouse complement (fig. 4c). Mouse CD59, on the other hand, was effective against both mouse and rat

complement (figs 4b and c). The data further indicates that rat, but not mouse CD59 is effective against human complement, since only transfectants expressing rat CD59 show an increase in resistance to human complement (fig. 4a). These data demonstrate the relative activities of each CD59 protein against heterologous sera, and data is relevant to establishing rodent models for the study of complement and complement inhibitors in tumor growth and control.

DISCUSSION

The phenomenon of homologous restriction, whereby cells are largely resistant to lysis by homologous complement, is due principally to the species selective function of CD59 and other membrane complement inhibitors [1]. However, species selective recognition of complement ligands is not absolute, and CD59 from different species vary in their effectiveness at inhibiting heterologous complement [12-14,17,18,29]. We show that human CD59, which is expressed on virtually all primary tumors and tumor cell lines that have been examined, is not effective against rat or mouse complement. We make use of this finding to demonstrate unequivocally that CD59 expressed on a human breast cancer cell provides efficient protection from complement-mediated lysis. Previous in vitro studies have shown that antibodies directed against complement regulatory proteins enhance susceptibility of tumor cells to complement-mediated lysis, and that isolated CD59 protects heterologous erythrocytes from human serum [2,3,5,9]. However, these studies do not exclude the possibility that other antibody- or CD59-interacting membrane molecules may effect complement function at the cell surface [31]. It is also possible that CD59 may provide functions other than direct protection from complement, and some data suggests a role for CD59 in cell signaling [33-35].

Previous in vitro data indicate that CD59 also provides cells with protection from the effects of sublytic MAC deposition [36]. Complement activation and sublytic MAC deposition on host cells

can trigger the release of various proinflammatory mediators, and can promote the expression of membrane vascular adhesion molecules involved in leukocyte recruitment [37-39]. These inflammatory processes may also play a role in host defense against tumor cells, and promoting their induction may further potentiate the effectiveness of immunotherapeutic approaches based on blocking CD59 function.

Our data indicate that endogenous CD59 expressed on human tumor cells implanted into rodents is unlikely to provide effective protection against complement attack when tumors are targeted by complement activating antibodies. The relative ineffectiveness of human CD59 against rat and mouse complement presents a serious hindrance for studies aimed at determining the protective role of CD59 (and other complement inhibitors) in rodent hosts bearing human cancers. The current data establishes the feasibility of using human cancer cells expressing rodent CD59 to show, in vivo, the regulatory effects of CD59 on complement-mediated tumor cell lysis. The aims of this study did not require that cell surface expression of rodent and (endogenous) human CD59 be quantitated relative to each other, although quantitative determinations of the activities of the various CD59 proteins against heterologous sera may provide insight into structure/function relationships of CD59 [18].

It is now clear that antibodies against cancer specific and overexpressed antigens are produced by patients [40]. However, identified endogenous anti-tumor antibodies do not appear to result in tumor destruction although deposition of complement may occur. Considered together with the high level of CD59 expression in primary tumors, it is reasonable to postulate that autologous anti-tumor antibodies elicited during tumor growth activate complement on some tumor surfaces, but that tumor cell lysis is prevented by tumor expressed complement inhibitors. Consequently, progressive tumor growth occurs. Inhibiting complement inhibitory proteins on a tumor cell surface may enhance the outcome of an endogenous tumor-specific cytolytic humoral immune

response, and may also greatly improve the outcome of anti-tumor immunotherapy using complement-activating monoclonal antibodies directed against a tumor antigen.

The targeted neutralization of CD59 on tumor cells in vivo presents a challenge since CD59 is widely expressed by normall tissue. Approaches for inhibiting complement inhibitors include the use of humanized antibodies that block function, or high affinity inhibitory-peptide mimetics. Possible methods for targeting and delivery include the use of encapsulated immunoliposomes or tumor-specific antibodies in techniques utilizing bispecific recognition of CD59 and tumor antigen [41,42]. Recently, the functional targeting of anti-CD59 antibodies to cancer cells by linking them with anti-tumor antibodies has been demonstrated in vitro[43,44].

In conclusion, our data strengthen the hypothesis that the modulation of CD59 activity on a tumor cell surface will provide an effective therapy when combined with complement-activating anti-tumor antibodies. Neutralization of CD59 (or other complement regulatory proteins) may also enhance a normally ineffective cytolytic humoral immune response. These hypotheses now need to be tested in vivo. To this end, the current data define important parameters necessary for establishing rodent models designed to evaluate the role of complement and CD59 in the growth and control of human cancer.

Acknowledgments This work was supported by grants AI34451(NIAID), CA66229 (NCI) and BC962437 (Department of the Army).

REFERENCES

1 Membrane defenses against attack by complement and perforins. Berlin: Springer-Verlag, 1992.

2 Hakulinen J, Meri S. Expression and function of the complement membrane attack complex inhibitor protectin (CD59) on human breast cancer cells. Lab Invest 1994; **71**:820-827.

3 Brasoveanu LI, Altomonte M, Gloghini A, et al. Expression of protectin (CD59) in human melanoma and its functional role in cell- and complement-mediated cytotoxicity. Int J Cancer 1995; **61**:548-556.

4 Bjorge L, Vedeler CA, Ulvestad E, Matre R. Expression and function of CD59 on colonic adenocarcinoma cells. Eur J Immunol 1994; **24**:1597-1603.

5 Yamakawa M, Yamada K, Tsuge T, et al. Protection of thyroid cancer cells by complement-regulatory factors. cancer 1994; **73**:2808-2817.

6 Varsano S, Frolkis I, Ophir D. Expression and distribution of cell-membrane complement regulatory glycoproteins along the human respiratory tract. Am J Resp Crit Care Med 1995; **152**:1087-1093.

7 Hofman P, Hsi BL, Manie S, Fenichel P, Thyss A, Rossi B. High expression of the antigen recognized by the monoclonal antibody GB24 on human breast carcinomas: a preventative mechanism of malignant tumor cells against complement attack? Breast Cancer Res Treat 1994; **32**:213-219.

8 Niehans GA, Cherwitz DL, Staley NA, Knapp DJ, Dalmasso AP. Human carcinomas variably express the complement-inhibitory proteins CD46 (membrane cofactor protein), CD55 (decay accelerating factor), and CD59 (protectin). Am J Pathol 1996; **149**:129-142.

9 Seya T, Hara T, Matsumoto M, Sugita Y, Akedo H. Complement-mediated tumor cell damage induced by antibodies against membrane cofactor protein. J Exp Med 1990; **172**:1673-1680.

10 Cheung N-KV, Walter EI, Smith-Mensah WH, Ratnoff WD, Tykocinski ML, Medof ME. Decay-accelerating factor protects human tumor cells from complement mediated cytotoxicity in vitro. J Clin Invest 1988; **81**:1122-1128.

11 Baranyi L, Baranji K, Takizawa H, Okada N, Okada H. Cell surface bound complement regulatory activity is necessary for the in vivo survival of KDH-8 rat hepatoma. Immunol 1994; **82**:522-528.

12 Rollins SA, Zhao JI, Ninomiya H, Sims PJ. Inhibition of homologous complement by CD59 is mediated by a species-selective recognition conferred through binding to C8 within C5b-8 or C9 within C5b-9. J Immunol 1991; **146**:2345-2351.

13 Tomlinson S, Wang Y, Ueda E, Esser AF. The expression and characterization of chimeric human/equine complement protein C9: localization of homologous restriction site. J Immunol 1995; **155**:436-444.

14 Huesler T, Lockert DH, Kaufman KM, Sodetz JM, Sims PJ. Chimeras of human complement C9 reveal the site of complement regulatory protein CD59. J Biol Chem 1995; **270**:3483-3486.

15 Seya T, Okada M, Hazeki K, Nagasawa S. Regulatory system of guinea-pig complement C3b: two factor I-cofactor proteins on guinea-pig peritoneal granulocytes. Biochem Biophys Res Comm 1990; **170**:514-512.

16 Kim YU, Kinoshita T, Molina H, et al. Mouse complement regulatory protein Crry/p65 uses the specific mechanisms of both human decay-accelerating factor and membrane cofactor protein. J Exp Med 1995; **181**:151-159.

17 Ish C, Ong GL, Desai N, Mattes MJ. The specificity of alternative complement pathway-mediated lysis of erythrocytes: a survey of complement and target cells from 25 species. Scand J Immunol 1993; **38**:113-122.

18 Yu J, Dong S, Rushmere NK, Morgan BP, Abagyan R, Tomlinson S. Mapping the regions of the complement inhibitor CD59 responsible for its species selectivity. Biochem 1997; **36**:9423-9428.

19 Rushmere NK, Harrison RA, van der Berg CW, Morgan BP. Molecular cloning of the rat analogue of human CD59: structural comparison with human CD59 and identification of a putative active site. Biochem J 1994; **304**:595-601.

20 Powell MB, Marchbank KJ, Rushmere NK, Van den Berg CW, Morgan BP. Molecular cloning, chromosomal localization, expression, and functional characterization of the mouse analogue of human CD59. J Immunol 1997; **158**:1692-1702.

21 Charreau B; , Cassard A, Tesson L, et al. Protection of rat endothelial cells from primate complement-mediated lysis by expression of human CD59 and/or decay-accelerating factor. Transpl 1994; **58**:1222-1229.

22 Harlow E, Lane D. Antibodies. A laboratory manual. New York: Cold Spring Harbor Laboratory, 1988.

23 Hughes TR, Piddlesden SJ, Williams JD, Harrison RA, Morgan BP. Isolation and characterization of a membrane protein from rat erythrocytes which inhibits lysis by the membrane attack complex of rat complement. Biochem J 1992; **284**:169-176.

24 Kinoshita T, Medof ME, Silber R, Nussenzweig V. Distribution of decay-accelerating factor in peripheral blood of normal individuals and patients with paroxysmal nocturnal hemoglobinuria. J Exp Med 1985; **162**:75-92.

25 Seya T, Hara T, Matsumoto M, Akedo H. Quantitative analysis of membrane cofactor protein (MCP) of complement. J Immunol 1990; **145**:238-245.

26 Davies A, Simmons DL, Hale G, et al. CD59, an Ly-6 protein expressed in human lymphoid cells, regulates the action of the complement membrane attack complex of homologous cells. J Exp Med 1989; **170**:637-654.

27 Yu J, Abagyan RA, Dong S, Gilbert A, Nussenzweig V, Tomlinson S. Mapping the active site of CD59. J Exp Med 1997; **185**:745-753.

28 Helfand SC, Hank JA, Gan J, Sondel PM. Lysis of human tumor cell lines by canine complement plus monoclonal antiganglioside antibodies or natural cannine xenoantibodies. Cell Immunol 1996; **167**:99-107.

29 Rushmere NK, Tomlinson S, Morgan BP. Expression of rat CD59: functional analysis confirms lack of species specificity and reveals that glycosylation is not required for function. Immunol 1997; **90**:640-646.

30 Xing PX, Tjandra JJ, Stacker SA, Teh JG, McLaughlin PJ, McKenzie IFC. Monoclonal antibodies reactive with mucin expressed in breast cancer. Immunol Cell Biol 1989; **67**:183-195.

31 Walsh LA, Tone M, Waldmann H. Transfection of human CD59 complementary cDNA into rat cells confers resistance to human complement. Eur J Immunol 1991; **21**:847-850.

32 Zhao J, Rollins SA, Maher SE, Bothwell ALM, Sims PJ. Amplified gene expression in CD59-transfected chinese hamster ovary cells confers protection agaisnt the membrane attack complex of human complement. J Biol Chem 1991; **266**:13418-13422.

33 Deckert M, Kubar J, Zoccola D, et al. CD59 molecule: a second ligand for CD2 in T cell adhesion. Eur J Immunol 1992; **22**:2943-2947.

34 Menu E, Tsai BC, Bothwell ALM, Sims PJ, Bierer BE. CD59 costimulation of T cell activation. J Immunol 1994; **153**:2444-2456.

35 van den Berg C, Cinek T, Hallett MB, Horejsi V, Morgan BP. Exogenous glycosyl phosphatidylinositol-anchored CD59 associates with kinases in membrane clusters on U937 cells and becomes Ca2+-signalling. J Cell Biol 1995; **131**:669-677.

36 Nangaku M, Meek RL, Pippin J, et al. Transfected CD59 protects mesangial cells from injury induced by antibody and complement. Kidney Int 1996; **50**:257-166.

37 Morgan BP. Complement membrane attack on nucleated cells: resistance, recovery and non-lethal effects. Biochemical Journal 1989; **264**:1-14.

38 Hattori R, Hamilton KK, McEver RP, Sims PJ. Complement proteins C5b-9 induce secretion of high molecular weight multimers of endothelial von Willebrand factor and translocation of granule membrane protein GMP-140 to the cell surface. J Biol Chem 1989; **264**:7768-7771.

39 Foreman KE, Vaporciyan AA, Bonish BK, et al. C5a-induced expression of P-selectin in endothelial cells. J Clin Invest 1994; **94**:1147-1155.

40 Canevari S, Pupa SM, Menard S. 1975-1995 revised anti-cancer serological response: biological significance and clinical implications. Ann Oncol 1996; **7**:227-232.

41 Holliger P, Winter G. Engineering bispecific antibodies. Curr Opin Biotech 1993; **4**:446-449.

42 Fanger MW, Morganelli PM, Guyre PM. Bispecific antibodies. Crit Rev Immunol 1992; **12**:101-124.

43 Junnikkala S, Hakulinen J, Meri S. Targeted neutralization of the complement membrane attack complex inhibitor CD59 on the surface of human melonoma cells. European Journal of Immunology 1994; **24**:611-615.

44 Harris CL, Kan KS, Stevenson GT, Morgan BP. Tumour cell killing using chemically engineered antibody constructs specific for tumour cells and the complement inhibitor CD59. Clin Exp Immunol 1997; **107**:364-371.

Figure legends

Fig. 1. Complement-mediated lysis of MCF7 cells. MCF7 cells were sensitized to complement by preincubation in the indicated concentrations of anti-MCF7 membrane rabbit antiserum. Sensitized cells were washed in media, exposed to 25% of either human, rat or mouse complement (37 °C/60 min), and cell lysis determined. The omission of either sensitizing antibody or of serum in cell lysis assays resulted in a background lysis of less than 10% of test value. Figure shows representative data from 3 separate experiments.

Fig. 2. Endogenous expression of complement inhibitory proteins by MCF7. Cells were stained by immunofluorescence using monoclonal antibodies to human CD59 (YTH53.1), MCP (M75), DAF (1A10), and CR1 (57F) as primary antibodies. Isotype matched antibodies of irrelevant specificity were used for controls. Relative fluorescence resulting from all control antibodies was below 12. Staining with a representative control antibody is shown. Histograms of the relative mean fluorescence intensities are shown.

Fig. 3. Expression of rodent CD59 by transfected MCF7. Stably transfected homogenous populations of MCF7 cells expressing either rat or mouse CD59 were isolated by several rounds of cell sorting. Figure shows flow cytometric analysis of sorted populations. Cells were stained by immunofluorescence using anti-rat CD59 monoclonal antibody (6D1) or rabbit anti-mouse CD59 polyclonal antibody. Note that Immunofluorescence is not quantitative relative to the different CD59 proteins. Histograms of the relative mean fluorescence intensities are shown.

Fig. 4. Complement-mediated lysis of MCF7 cells expressing rodent CD59. Control and transfected MCF7 cells were sensitized to complement by preincubation in 15% anti-MCF7 antiserum. Sensitized cells were exposed to different concentrations of either human (a), rat (b) or mouse (c) serum, and lysis determined. The omission of either sensitizing antibody or of serum in

cell lysis assays resulted in a background lysis of less than 10% of test value. Figure shows representative data from 3 different experiments.

Fig. 1

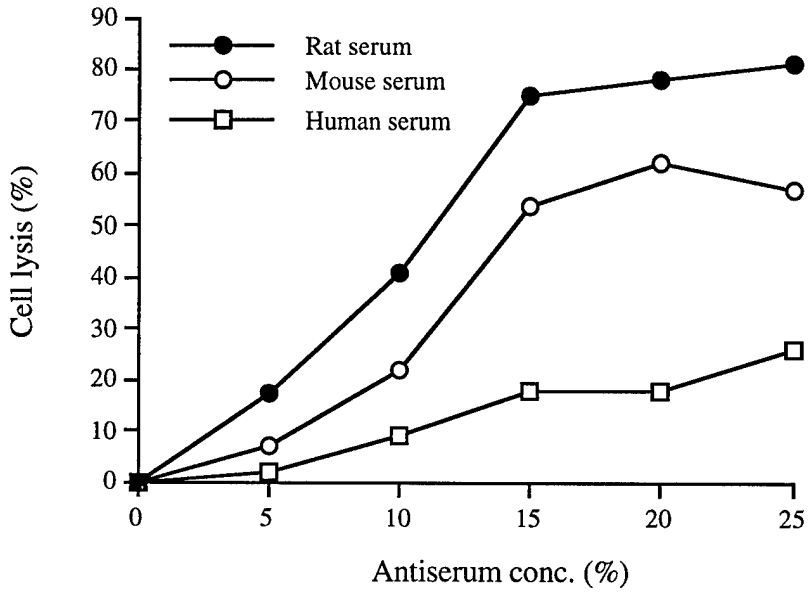
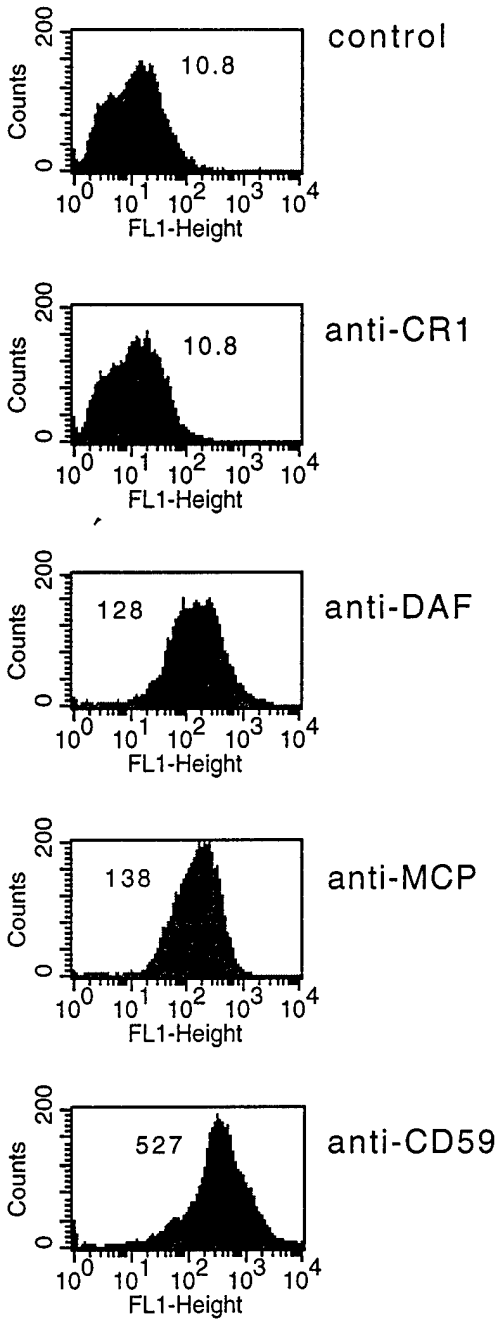
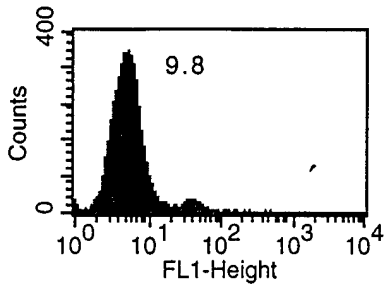

Rat serum
Mouse serum
Human serum
Cell lysis (%)
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Antiserum conc. (%)

Fig. 2

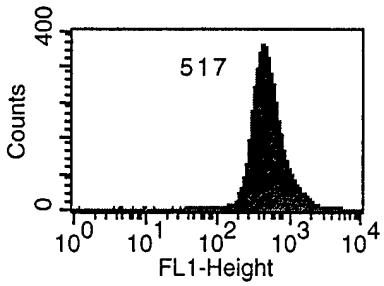

control
10.8
anti-CR1
10.8
anti-DAF
128
anti-MCP
138
anti-CD59
527
Counts
200
0
10^0 10^1 10^2 10^3 10^4
FL1-Height

anti-rat CD59

Untransfected MCF7

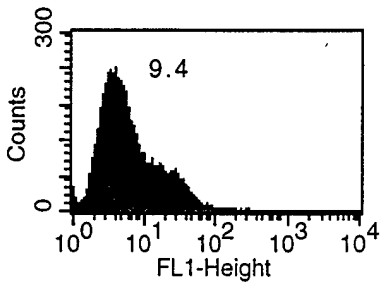


MCF7-Rat CD59



anti-mouse CD59

Untransfected MCF7



MCF7-Mouse CD59

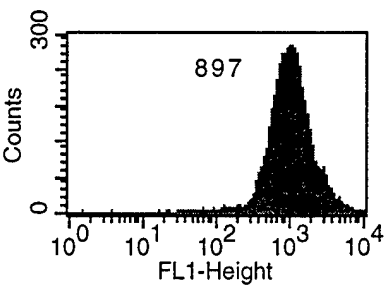
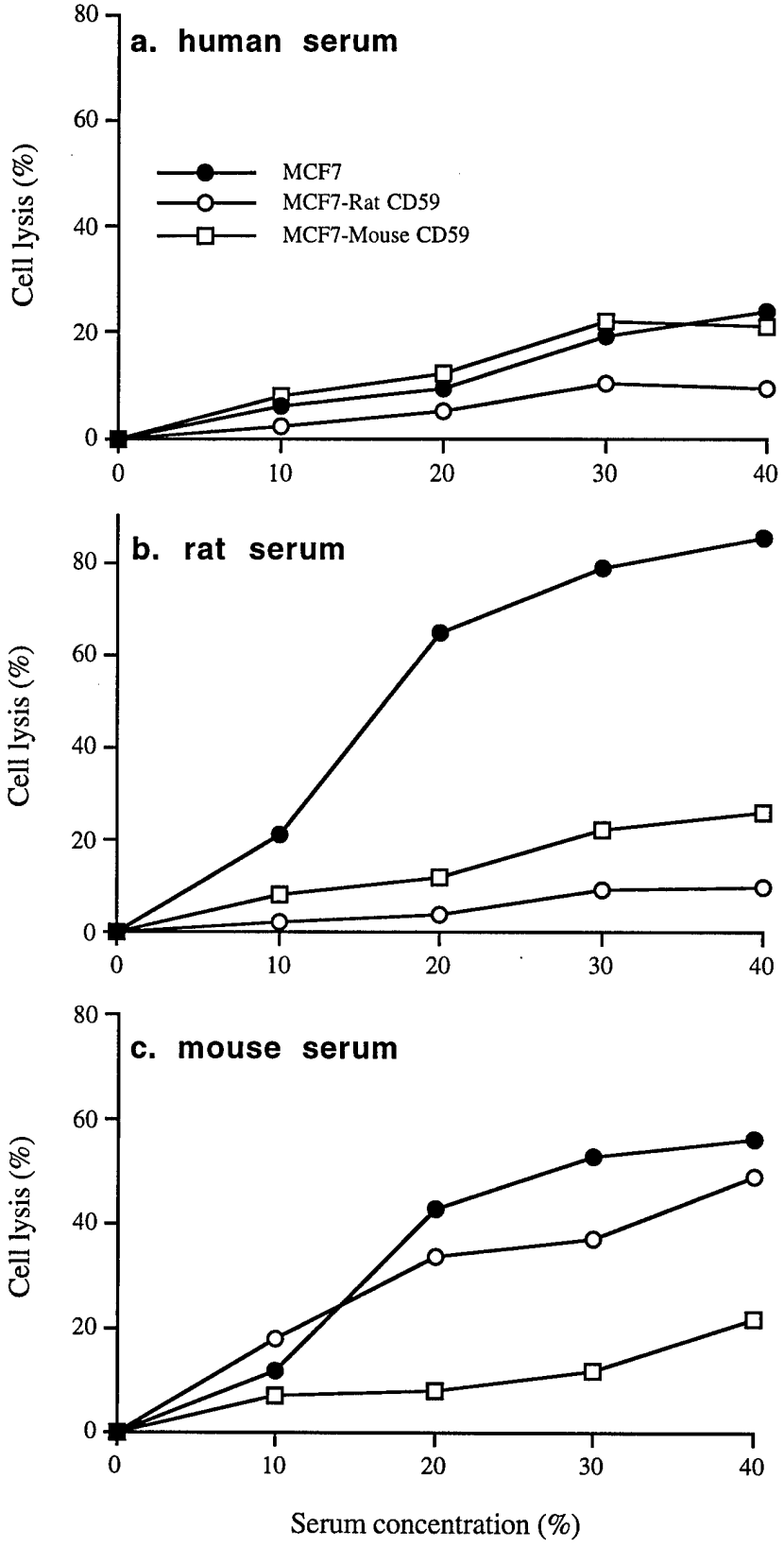


Fig. 4


a. human serum
b. rat serum
c. mouse serum
MCF7
MCF7-Rat CD59
MCF7-Mouse CD59
Cell lysis (%)
Serum concentration (%)
0
20
40
60
80
10
30